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Catalytic mechanism and product specificity of cyclodextrin glycosyltransferase, a prototypical transglycosylase from the α-amylase family

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Abstract

The catalytic mechanism of cyclodextrin glycosyltransferase, a member of the α-amylase family, is reviewed. The focus is put on the bond cleavage mechanism, the nature of the transition state and of the covalent intermediate, and on the stereo-electronic and lateral protonation contributions to catalysis. The functions in catalysis of the absolutely conserved residues in this family are discussed. Finally, the fascinating capability of cyclodextrin glycosyltransferase to produce cyclodextrins from linear starch oligosaccharide chains is reviewed, together with protein engineering studies to modify the enzyme's product specificity.

1. Introduction

Life on earth depends on the uptake of energy. While photosynthetic organisms can directly use the sun's light, all other forms of life obtain their energy from nutrients they take up from the environment. One of the most energy-rich sources of food is starch, which is a polymer of glucose molecules linked together by α(1→4) and α(1→6) glycosidic bonds [1,2]. It is consumed daily by a large part of earth's human population in the form of rice, wheat, maize, tapioca, or potatoes.

To degrade starch humans, but also other animals, fungi and bacteria possess a range of starch-degrading enzymes. Most important in this respect is a large and diverse group of hydrolytic enzymes, which have been classified in glycosyl hydrolase family 13, the α-amylase family [3,4]. This family comprises not only specialized enzymes for the hydrolysis of α(1→4) and α(1→6) glycosidic bonds, but also enzymes that make oligosaccharide products of a specific composition.

An example of such an enzyme that produces specific oligosaccharides is cyclodextrin glycosyltransferase (CGTase). CGTase is a 75 kDa bacterial enzyme that converts starch into cyclodextrins, which are cyclic oligoglucosides of six, seven or eight glucose residues (named α-, β-, and γ-cyclodextrin, respectively). Presumably, the bacteria that excrete CGTases do this to convert starch into compounds that can not be used by competing organisms. In this way they monopolize starch as a substrate for themselves [5].

However, besides its obvious functional importance for the bacteria that excrete it, cyclodextrin glycosyltransferase has also found important industrial applications. The major interest in the use of CGTase is for the large-scale production of cyclodextrins, but the enzyme is also applied for the liquefaction of starch [6]. A more recent development is the use of CGTase for the synthesis of modified oligosaccharides, such as the sweetener stevioside [6]. Stevioside, as isolated from the plant *Stevia rebaudiana*, has bitter taste and low solubility. CGTase catalyzed glycosylation decreases the bitterness and increases the solubility.

Despite its wide use in industry, CGTase suffers from several shortcomings that limit its applicability. All wild type CGTases produce mixtures of α-, β-, and γ-cyclodextrins, and the separate cyclodextrins have to be purified by selective crystallization using (expensive) organic solvents, that are sometimes difficult to remove. A second disadvantage of the CGTases commonly used in industry is that their activity is inhibited by the produced cyclodextrins [7]. Thus, there is a clear need for novel CGTases with improved

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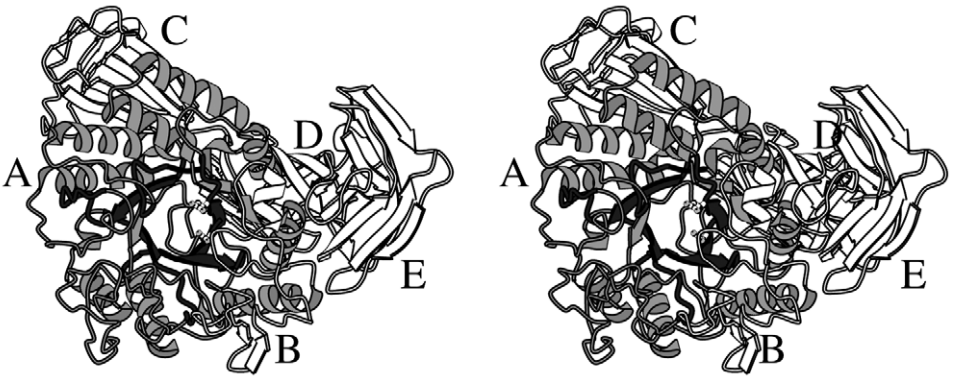

Fig. 1. Stereo-view of the structure of a representative member of the α-amylase family, *Bacillus circulans* strain 251 cyclodextrin glycosyltransferase (BC 251 CGTase) [11]. CGTase is a 75 kDa enzyme that consists of five domains. Domain A is the catalytic $(\beta/\alpha)_8$ barrel (gray), that CGTase shares with the other members of the α-amylase family. This domain contains the sequence regions that are conserved in the α-amylase family (black). In the center of the conserved regions indicated by the side chains of Asp229 and Glu257 in white ball-and-stick representation, the catalytic site is located. From domain A a loop protrudes that forms part of the active site (white; domain B), and which is also present in many other α-amylase family enzymes [18]. Behind the $(\beta/\alpha)_8$ barrel, three additional domains (C, D and E) are present (white). Domains C and E contain binding sites for raw starch [7,11]. This figure, and Fig. 4, were made with Molscript [74].

properties. In this article we review the existing literature on the catalytic mechanism of CGTase and related enzymes, and our protein engineering approach to improve the product specificity of the enzyme.

2. Three-dimensional structures of cyclodextrin glycosyltransferase

When in 1984 the first X-ray structure of an α-amylase family member, Taka-α-amylase, was elucidated, this revealed a catalytic domain with an $(\alpha/\beta)_8$, or TIM barrel, architecture [8]. At present 22 X-ray structures of α-amylase family members are known, among which are several of cyclodextrin glycosyltransferases [9–13]. CGTases have five domains, labeled A to E (Fig. 1). Domain A is the catalytic $(\alpha/\beta)_8$ domain, which CGTase has in common with the other α-amylase family members. Domain B is an extended loop region inserted after β-strand 3 of domain A. It contributes to substrate binding by providing several amino acid side chains alongside a long groove on the surface of the enzyme that interact with the substrate [14,15]. Domains C and E have a β-sheet structure, and are specialized in binding to raw starch granules [7,11,16]. The function of domain D, which also has a β-sheet structure, remains to be elucidated.

The seven most conserved amino acid residues in the α-amylase family cluster together at the bottom of the substrate binding groove at the surface of the $(\alpha/\beta)_8$ barrel. They are in the center of the conserved regions (Fig. 1), where the catalytic site is located. Three of them are absolutely conserved, Asp229, Glu257 and Asp328, while Asp135, His140, Arg227 and His327 are almost completely conserved [3,17,18]. The evolutionary conservation of these residues can be explained because their common substrate, starch, contains only one basic chemical bond, the α-glycosidic bond. This bond occurs in several types, like $\alpha(1\rightarrow4)$, $\alpha(1\rightarrow6)$ or $\alpha(1\rightarrow1)$, depending on whether the C1 atom of a glucose is connected to the O4, O6 or O1 atom of a neighboring glucose, respectively (see Fig. 2, left part, for the glucose ring atom nomenclature).

α-Glycosidic bonds are very stable, having a rate of spontaneous hydrolysis of only about $2 \cdot 10^{-15}\ s^{-1}$ at room temperature [19]. Since for example CGTase has a rate in

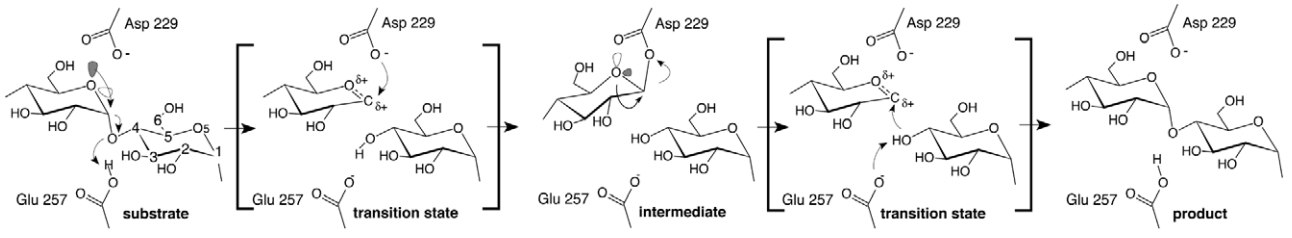

Fig. 2. Scheme of the α-retaining $\alpha(1,4)$ glycosidic bond cleavage mechanism as used by the α-amylase family, and as explained in the text. The glycoside ring atom nomenclature is incorporated in the left-most picture. The shaded orbitals represent the electrons that participate in the cleavage of the α-glycosidic bond according to the stereo-electronic theory (see text). Note that both the substrate and the product contain an identical $\alpha(1,4)$ glycosidic linkage. In the disproportionation, cyclization, and coupling reactions the part of the substrate that is not covalently bound to the enzyme during the reaction, is exchanged for a different saccharide, but the newly formed glycosidic bond remains an $\alpha(1,4)$ glycosidic bond.

the order of 3000 s^{-1} [20], this means that CGTase enhances the hydrolysis rate by 18 orders of magnitude, which ranks the enzyme among the most efficient ones known [19].

3. The reactions catalyzed by cyclodextrin glycosyltransferase

CGTase catalyzes four distinctly different reactions: three transglycosylation reactions (disproportionation, cyclization, and coupling), and a hydrolysis reaction [20]. Disproportionation is the major reaction. In this reaction a linear malto-oligosaccharide is cleaved and one of the products is transferred to another linear acceptor substrate. Cyclization is an intramolecular transglycosylation reaction, in which the non-reducing end of a linear malto-oligosaccharide is transferred to the reducing end of the same oligosaccharide chain. The products of this reaction are cyclodextrins. Coupling is the reverse of the cyclization reaction in which the opening of a cyclodextrin ring is followed by transfer to a linear oligosaccharide. Finally, hydrolysis is a reaction in which part of a linear oligosaccharide is transferred to water. These four reactions make use of the same basic mechanism, which is conserved throughout the α-amylase family; only the acceptor molecule differs. In the following we will use the standard sugar binding subsite nomenclature [21], in which the catalytic site is labeled -1, and the scissile bond is positioned between subsites -1 and $+1$. In this nomenclature the acceptor molecule will bind at the $+1$ subsite, after the bond cleavage reaction has taken place.

4. The bond cleavage mechanism of cyclodextrin glycosyltransferase

The catalytic mechanism of enzymes of the α-amylase family, which is now generally accepted, was first proposed by Koshland in 1953 [22], and is known as the α-retaining double displacement mechanism. This mechanism involves two catalytic residues, an acid/base catalyst (Glu257 in CGTase) and a nucleophile (Asp229) (Fig. 2). When substrate has bound, Glu257 (as acid) donates a proton to the scissile glycosidic bond oxygen. The scissile bond is then cleaved, during which reaction the -1 sugar goes through an oxocarbonium ion-like transition state (Fig. 2). This transition state is characterized by a partial double bond between the O5 and C1 atoms of the sugar over which a positive charge has delocalized. It has a planar configuration of the C2, C1, O5 and C5 atoms (Fig. 2).

In the subsequent reaction step, this oxocarbonium ion-like transition state collapses into a stable covalent glycosyl-enzyme reaction intermediate which is β-glycosidically linked to the nucleophile Asp229 (Fig. 2). The leaving group (at subsite $+1$) is exchanged for a new compound, the acceptor, which gets activated by Glu257 (now a base). This activated acceptor attacks the covalent intermediate, and, via another oxocarbonium ion-like transition state, the final α-glycosidic product bond is formed (Fig. 2). This α-retaining mechanism is closely related to the β-retaining mechanism of glycosyl hydrolases that act on β-glycosidic bonds. Both mechanisms have been extensively reviewed [23–29].

5. The nature of the transition state

For the α-amylase family enzymes, the transition state has been characterized by kinetic isotope experiments [30]. These studies have shown that it has an almost complete double bond between the O5 and C1 atoms, with the positive charge almost entirely localized on the O5 atom, the O4′ atom nearly fully protonated, and the C1–O4′ bond almost completely broken [24,30]. Thus, these measurements confirm an oxocarbonium ion-like structure. Such a transition state is further supported by the fact that the functional unit of the transition state mimicking inhibitor acarbose has a planar geometry, similar to that of an oxocarbonium ion [31].

6. The nature of the intermediate

The chemical nature of the intermediate has been the subject of an almost classical debate [26,32]. In his original proposal, Koshland drew the intermediate in α- and β-retaining mechanisms as covalent [22]. However, when the first structure of a glycosyl hydrolase was solved, the β-retaining hen egg white lysozyme, it was suggested that an oxocarbonium ion might be sufficiently long-lived in the catalytic site to function as intermediate [33]. This view became widespread in textbooks [1], and was readily extrapolated to the α-amylase family enzymes [3,8,11,34].

The first unambiguous experimental evidence for the existence of a covalent intermediate in the α-amylase family came from NMR studies [35], and trapping experiments using radiolabels [36]. Furthermore, experiments to trap the intermediate with inhibitors and slow substrates were successful [37–41]. However, the most convincing evidence for the covalent character of the intermediate was obtained from a 3D structure of a trisaccharide covalently bound to the nucleophile Asp229 in CGTase [42]. Owing to this evidence, covalent intermediates are at present widely believed to occur in the α-amylase family, thereby confirming Koshland's original hypothesis. Very recently unambiguous evidence has been provided that also hen egg white lysozyme makes use of covalent catalysis [23–29].

7. Stereo-electronic contributions to catalysis

A second important field of debate concerns the predictions of the stereo-electronic theory about the conformations

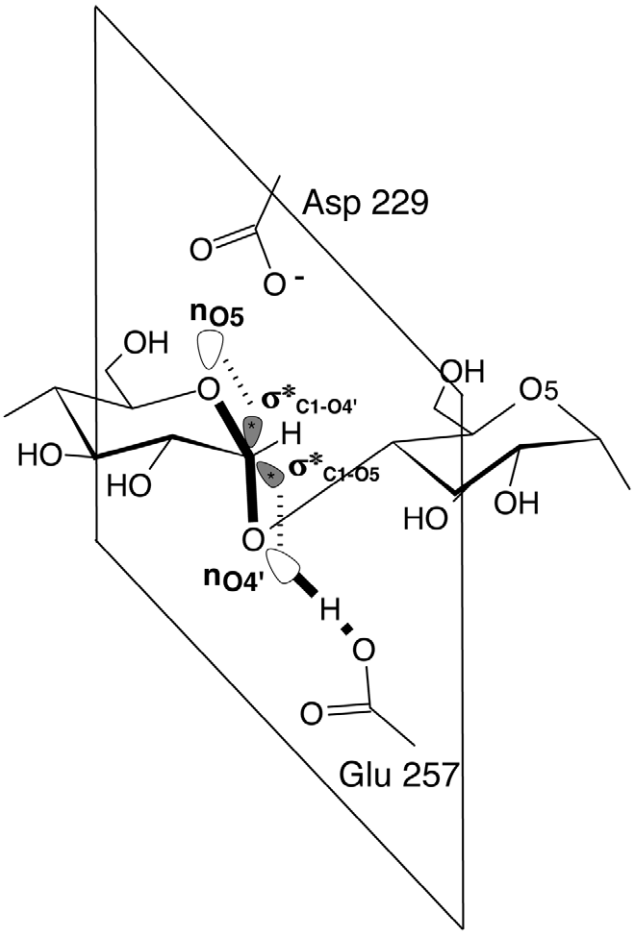

Fig. 3. The α(1,4) glycosidic bond orientation in a substrate bound in the active site of an α-amylase family enzyme. Lone pair electron orbitals are shown in white, anti-bonding orbitals in gray. Dashes indicate orbital overlap. Also depicted is the hydrogen bond that Glu257 donates to the lone pair electron orbital of the scissile bond oxygen ($n_{O4'}$). The bonds that are involved in the reaction (black) are oriented in one plane, which is represented by the frame (see text). The glucose on the left has a $^4C_1$ chair conformation, in which case the α-glycosidic bond assumes an axial orientation.

of substrate and intermediate when bound to the enzyme [23,43]. The stereo-electronic theory is based on the (undebated) anomeric effect, which explains the unexpectedly high preference of a free glucose molecule for having its C1–O1 bond in an axial, instead of an equatorial orientation [44]. The anomeric effect proposes a stabilizing interaction between a non-bonding lone pair orbital of the O5 atom (n_{O5}), and the anti-bonding orbital of a C1–O1 bond or a C1–O4′ bond ($\sigma^*_{C1-O4'}$), when this bond has an axial orientation [23,45] (Fig. 3). This orbital overlap leads to a shortening and strengthening of the O5–C1 bond [23,46], and an elongation and weakening of the C1–O4′ bond. This might be advantageous for enzymes, and therefore it has been stated that glycosidic bonds are preferentially cleaved when they are in an axial position.

For α-glycosidically linked sugars, the glycosidic bond is in an axial position when the sugar is in a $^4C_1$ chair-like conformation (Fig. 3). Indeed, in X-ray structures of enzymes in complex with an α-glycosidically linked substrate [16], or an α-glycosidically linked intermediate [47], these ligands are observed in a $^4C_1$ chair-like conformation. The stereo-electronic theory further predicts that β-glycosidically linked substrates and intermediates are distorted towards a ^{1,4}B boat conformation to impose an axial orientation of the scissile bond. This distortion is indeed observed in a complex of an enzyme with its β-glycosidically linked substrate [48]. However, in a β-glycosidically linked covalent intermediate, as occurs in the α-amylase family, such distortion is not observed [42].

Therefore, not all X-ray structures confirm the stereo-electronic theory. Furthermore, an alternative explanation has been put forward for the distortion of a β-glycosidically linked substrate towards a ^{1,4}B boat conformation, stating that this boat conformation might be required to minimize steric hindrance during the enzymatic reaction [49]. Moreover, the $^4C_1$ chair-like conformations in α-glycosidically linked sugars have low energy, and might thus simply be thermodynamically favored. For these reasons, the stereo-electronic theory has fallen out of favor recently [49].

8. The contribution of lateral protonation to catalysis

A third interesting issue in the field of catalysis by glycosyl hydrolases is the function of distortions of glycosidic bond torsion angles. Often the binding of a substrate in the active site of a glycosyl hydrolase results in rotations of the torsion angles around the C1–O4′ and O4′–C4′ bonds, and the disruption of an interglycosidic hydrogen bond between the “intermediate” and “leaving group” parts of the substrate. Therefore, it was suggested that these torsion angle rotations facilitate departure of the leaving group after formation of the intermediate [50,51]. However, the rotations also position a lone pair orbital of the O4′ atom in the direction of the proton donor Glu257 (Fig. 3). This allows lateral protonation of the glycosidic oxygen atom, in which the O5–C1 bond, the C1–O4′ bond, and the “line of protonation” between O4′ and Glu257 occur in one plane (Fig. 3) [27,52]. Fulfilling a requirement for lateral protonation might thus also be a function for the bond torsion angle distortions, although the reason for lateral protonation remains unknown. An analogous requirement for an in-plane orientation of the reactive system is seen in E2-elimination reactions [44]. In both cases it might serve to suitably orient the reactive orbitals for a bond cleavage reaction.

9. The roles of the conserved residues in catalysis

Asp229 is the first absolutely conserved residue in the α-amylase family, and the proposed catalytic nucleophile (Fig. 2). Mutations in Asp229 reduce the activity about ~25,000 times [3,16,18]. In structures of ligand complexes

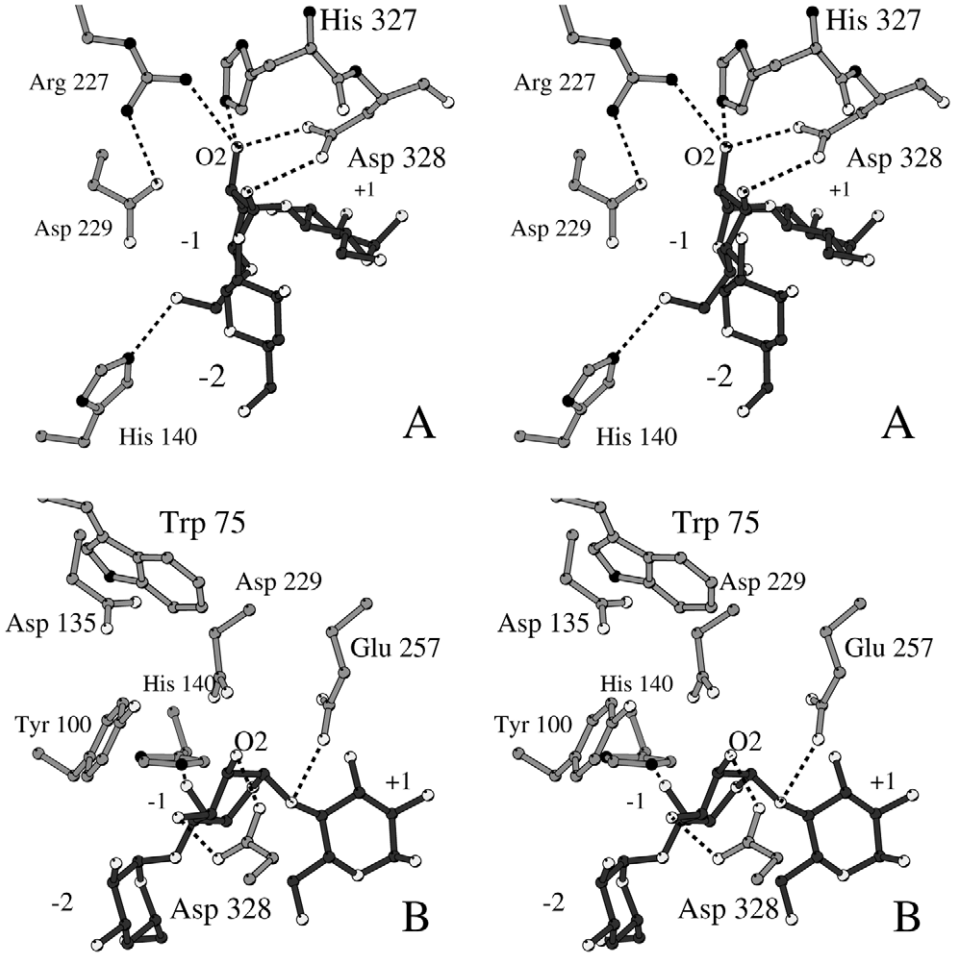

Fig. 4. Stereo-view of the conserved residues in the catalytic site (gray) of the α-amylase family in two orientations (A and B). For clarity, a substrate molecule (black) spanning subsites −2, −1 and +1 is also shown. Dashed lines indicate hydrogen bonding interactions. Asp328 makes hydrogen bonds with the O2 and O3 atoms of the −1 glucose, His140 is hydrogen bonded to the −1 O6 atom, and the Glu257 side chain is at hydrogen bonding distance from the oxygen atom in the scissile glycosidic bond. The figure is based on a BC251 CGTase-substrate complex described in reference [42], and is essentially similar to substrate complexes of other α-amylase family enzymes.

(Fig. 4), the Oδ1 atom of Asp229 is situated close to the glucose C1 atom, and its reactive *syn* lone pair orbital is positioned ideally for nucleophilic attack [53,54].

Glu257 is the second absolutely conserved residue in the α-amylase family, and the proposed catalytic acid/base (Fig. 2). Mutation of Glu257 reduces the enzymatic activity ∼5,000 times [3,16,18]. In structures of unliganded α-amylase family enzymes, Glu257 receives a hydrogen bond from Asp328. After sugar binding, the side chain of Glu257 rearranges (Fig. 4), and forms a hydrogen bond to the O4′ atom in the scissile bond [53], as expected for the catalytic acid/base residue [8].

Asp328 is the third absolutely conserved residue. Mutants in Asp328 can reduce the activity ∼50,000 times [3,16]. Upon substrate binding Asp328 interacts with the OH-2 and OH-3 groups of the substrate [8,53,54] (Fig. 4). It has been speculated that the hydrogen bond to the OH-2 group could become a strong low-barrier hydrogen bond that stabilizes the transition state of the reaction [54]. It has also been suggested that Asp328 plays a role in maintaining the proper protonation state of Glu257 [53]. Finally, Uitdehaag et al. demonstrated that Asp328 has an important role in substrate distortion [42].

His327 and Arg227 are two basic residues in the catalytic site. Mutants of His327 typically show a ∼50 times reduced k_{cat}/K_M [3,28,55,56]. Mutants of Arg227 have not been reported. His327 and Arg227 both bind to the OH-2 group of the substrate [57] (Fig. 4). These hydrogen bonds serve two purposes. One is the distortion of the sugar ring towards the half-chair conformation. The other is a reduction of the electronegativity of the 2-hydroxyl group, which otherwise would strongly disfavor formation of a positively charged transition state [42].

His140 is located further down in the catalytic site. Mutants of this residue have a ∼200 times reduced k_{cat}/K_M [3,28,55,56]. X-ray structures show that His140 binds to the OH-6 group of the −1 sugar of a substrate bound in the catalytic site [42] (Fig. 4). However, in the CGTase-cova-

lent intermediate and the CGTase-γ-cyclodextrin product complex this hydrogen bond is absent, and His140 interacts with the Asn139 side chain [15,42]. Thus, the nature of the His140–OH-6 hydrogen bond most likely changes during catalysis [15,58].

Asp135 is also strongly conserved in α-amylases. However, this residue is not located in the catalytic site. Instead, it binds to the catalytic site residue Tyr100, which is less strongly conserved (Fig. 4). The role of Asp135 is unknown, but Tyr100 has a function in sugar binding. Its aromatic ring interacts with the glucose ring in the catalytic subsite (glucose −1) through hydrophobic stacking contacts [53, 57].

10. Cyclization activity of cyclodextrin glycosyltransferase

A fascinating feature of cyclodextrin glycosyltransferase is its capability to produce cyclodextrins from linear starch oligosaccharide chains. Cyclization activity is a special form of acceptor specificity, in which the non-reducing end of a donor chain is used as acceptor, leading to cyclic products (see Fig. 5). In *Bacillus circulans* strain 251 CGTase, which produces predominantly β-cyclodextrin, seven sugar binding subsites were characterized that bind the covalent intermediate (donor subsites) [14,42]. Surprisingly, in the crystal structure, the 4-OH group at the non-reducing end of the substrate, bound at subsite −7, was about 23 Å away from the catalytic site, and not nearby, as originally expected [59]. Subsequent site-directed mutagenesis studies confirmed the remote location of subsite −7, assuring that its identification is not a crystallographic artifact [60,61]. Thus, cyclization requires a step in which a linearly bound covalent intermediate, reaching subsite −7, changes conformation into a circularly bound intermediate, reaching the acceptor subsite +1. Using stochastic path calculations, Uitdehaag et al. analyzed the pathway along which the non-reducing end of the covalent intermediate moves from subsite −7 to the +1 acceptor subsite [62]. The input data for these calculations were the structure of a linearly bound malto-octaose saccharide, derived from the crystal structures of CGTase with bound maltononaose and with the covalently bound maltotriose [42], and the structure of γ-cyclodextrin, bound in the active site of CGTase [15]. The result is one of the first successful applications of the method. Comparison of the results with site-directed mutagenesis data show that CGTase actively catalyzes the circularization process. The −6, −7, −8 sugar residues successively bind at subsite −6, where they interact with Gly179, Gly180, Tyr167, and Asn193. Further movement of the oligosaccharide is facilitated by interactions with the aromatic side chains of Tyr195, Phe183, and Phe259. Next, residues Leu194, Leu197 and Ala230, assisted by Tyr195 and Phe183, form a hydrophobic cavity that traps the sugar chain before its final transfer to the +1 acceptor subsite. During the circularization process the sugar binding mode at subsite −3 changes; the initial interactions with Asp196 and Tyr89 weaken, and the saccharide binds instead to Arg47 and Asp371. An animation of this circularization process can be found at http://www.interscience.wiley.com/jpages/0887-3585/suppmat/43_3/v43_3.html.

11. Protein engineering of the product specificity of CGTase

In the past it has been suggested that the size of the aromatic amino acid (Tyr195), which is present in a central position in the active site, is an important determinant for the size of the produced cyclodextrins. Indeed, a Tyr195Trp substitution doubled the amount of γ-cyclodextrin [60,63]. However, several other mutations of this residue did not support these results [63–65]. In the light of the recent findings of Uitdehaag et al. on the circularization mechanism (see above, [62]) it is now clear that the outcome of the cyclization reaction is determined by many more factors than only residue 195. Instead, interactions at subsites −7, −6, and −3 may all contribute to the product specificity of CGTase.

The importance of subsites −7 and −3 for cyclodextrin product specificity is supported by a large body of evidence. The primarily γ-cyclodextrin producing CGTase from *B. firmus/lentus* strain 290-3 completely lacks the residues around residue 146 (subsite −7). This situation was recently copied into the CGTase from *B. circulans* strain 8, by replacing residues 145–151 by a single aspartate residue. The mutant had an increased γ-cyclodextrin production [60]. Similarly, a Ser146Pro mutation in *Bacillus circulans* strain 251 CGTase (subsite −7) resulted in a lower β-cyclization, but a higher α-cyclization activity [61].

At subsite −3 Tyr89 plays an important role. The Tyr89Asp substitution in *Bacillus circulans* strain 251 CGTase showed a slight increase in α-cyclization activity, with no effect on the β- and γ-cyclization activity [61]. Combination of this mutation with the subsite −7 Ser146Pro substitution gave an enzyme that produced significantly more α-cyclodextrin than wild type enzyme [61]. Mutation of Arg47 near subsite −3 into a Leu or Gln resulted in a generally decreased cyclization activity, but also a shift towards the production of larger cyclodextrins [66]. Finally, structural investigations of the CGTase from *Thermoanaerobacterium thermosulfurigenes* EM1 [12,67] revealed the importance of Asp371 and Asp197 at subsite −3 for product specificity. Mutant Asp371Arg produced enhanced levels of β- and γ-cyclodextrin, while mutant Asp197His yielded more α-cyclodextrin [67]. Subsite −3 mutations in the alkalophilic *Bacillus* sp. I-5 CGTase also changed the product specificity [68].

From the above it is clear that subsites −3 and −7 are key sites for the cyclodextrin product specificity. The identity, position and interactions of the amino acid residues

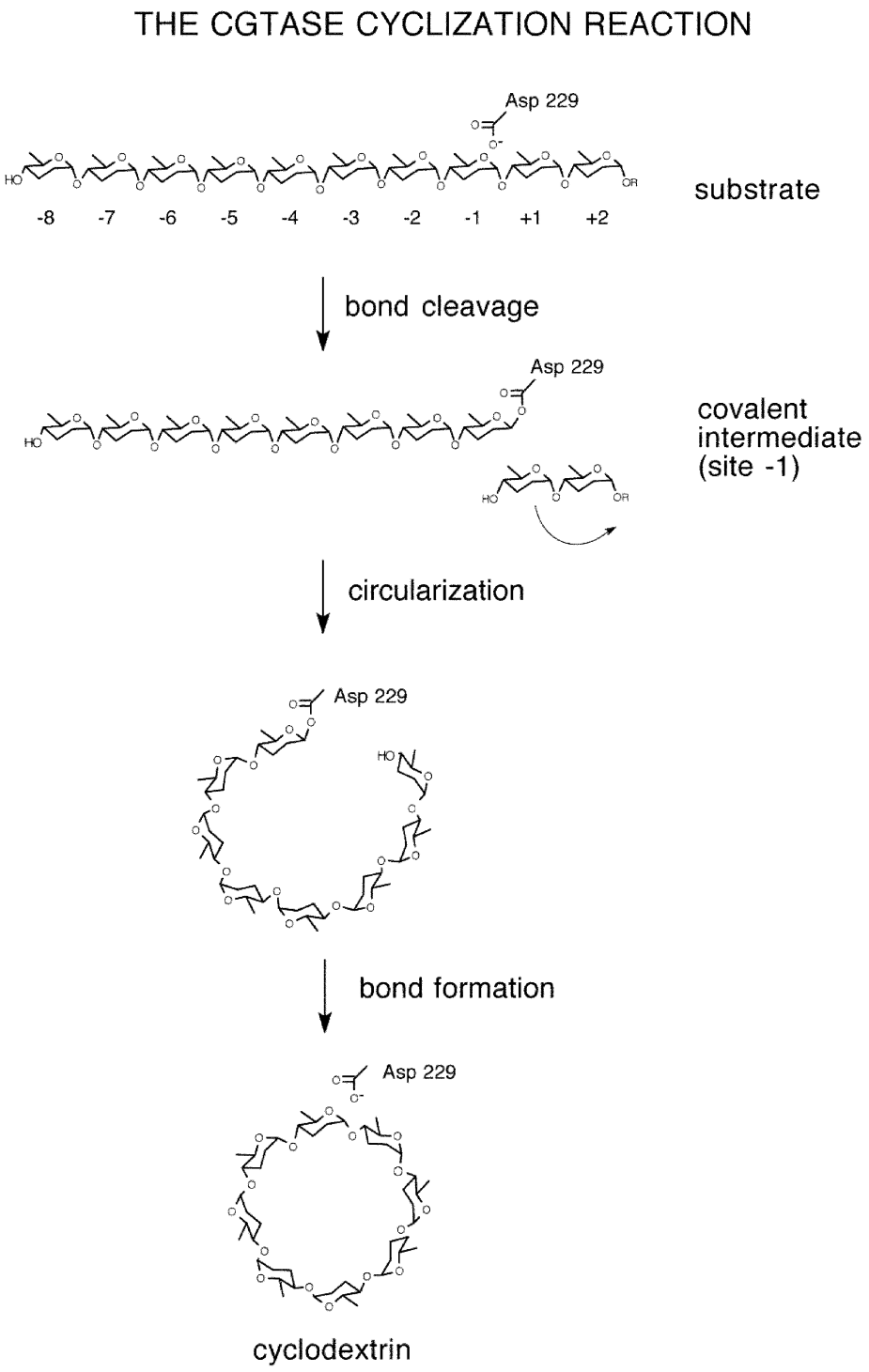

Fig. 5. Scheme of the cyclization reaction of CGTase. After the first, bond-cleavage step, a covalent intermediate is formed. In the second step the linear chain first assumes a cyclic conformation, which is the circularization step. Subsequently, a product bond is formed with the terminal 4-OH group of the intermediate. The catalytic residues involved in bond cleavage are Asp229 and Glu257 (the latter is not shown for clarity).

responsible for this product specificity have been identified. This detailed insight now allows the rational construction of mutant CGTase enzymes with desired cyclodextrin product specificity [69].

12. Future prospects

The results from a worldwide research effort to understand the mechanism of action of cyclodextrin glycosyl-

transferase have now yielded insights that can be used in a rational way to tailor CGTases for specific applications [69–71]. Nevertheless, there is much more to understand. Uitdehaag et al. [15,58] obtained structural evidence for three distinctly different conformational states of the CGTase active site in its interaction with substrates, which suggests an induced fit mechanism that could explain the high transglycosylation activity of the enzyme. However, how substrate binding induces these conformational changes, and to what extent and via which mechanism the conformational rearrangements affect activity, is far from understood. Furthermore, the cause of the low hydrolytic activity of CGTase has remained obscure so far, as well as the role of the raw starch binding-sites in the processivity of starch degradation and the regulation of the enzyme's activity [7]. The combined use of in depth enzyme kinetics and mutation studies, together with structural studies and careful reaction pathways analyses, and possibly supplemented by directed evolution approaches [72,73] may be a valuable approach to better understand the catalytic properties of cyclodextrin glycosyltransferase.

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